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Osmoregulation in the Halotolerant Alga *Asteromonas gracilis*

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ABSTRACT

***Asteromonas gracilis*, a green wall-less halotolerant alga, grows on salt concentrations from 0.5 molar NaCl (seawater) to saturation (4.5 molar NaCl). The specific growth rate was maximal at concentrations between 0.5 and 2.5 molar and only gradually decreased above 2.5 molar. Photosynthetic oxygen evolution was maximal over a range of salinities around 2.5 molar and the photosynthesis to respiration ratio showed a maximum at 1.5 molar NaCl. The alga accumulates large amounts of intracellular glycerol in response to saline conditions. The glycerol content of the cells varied in direct proportion to the extracellular salt concentration, being about 50 and 400 picograms glycerol per cell in algae grown at 0.5 and 4.5 molar NaCl, respectively. In salt concentrations lower than 3.5 molar and at growth temperatures below 40 C, essentially all the glycerol was intracellular. Above 3.5 molar NaCl, about 25 per cent of the total glycerol leaked slowly from the cells to the medium. Treating the algae for several minutes at temperatures exceeding 47 C caused 50 per cent release of the internal glycerol. At 60 C, 100 per cent of the glycerol was released. When the extracellular salt concentration was increased or decreased, the intracellular glycerol varied accordingly, reaching its new intracellular level after a few hours. Both photosynthesis and respiration were inhibited on transfer of the cells from 1.5 to 3.5 molar NaCl but were not inhibited on transfer of the cells from 3.5 to 1.5 molar NaCl. The kinetics of photosynthetic resumption preceded the kinetics of glycerol biosynthesis. The above results indicate the existence of osmotic regulations in *Asteromonas gracilis* via the accumulation of intracellular glycerol.**

Asteromonas gracilis Artari is a green wall-less unicellular flagellate of the class Prasinophyceae present in salt marshes and small brine ponds (10, 15, 17, 19, 20). The algae are often subjected to widely fluctuating salt concentrations and can tolerate a broad range of salt, from low salinity to saturated NaCl solutions. In contrast to the many studies which were undertaken on the halotolerant alga *Dunaliella* (9) to elucidate the mechanism of its osmotic adaptation under salt stress (2, 4–8), the information on osmoregulation in *Asteromonas* is rather limited. Recently Ben-Amotz and Avron (5) showed that *Asteromonas* and *Dunaliella* produce and accumulate glycerol to a concentration isoosmotic with the medium and, thus, are able to survive in saline water. The communication presented here describes an investigation of the physiological behavior of *A. gracilis* under salt stress.

MATERIALS AND METHODS

Algae. *A. gracilis* Actari 80/1 was obtained from the Culture Collection of Algae and Protozoa, Cambridge, England, and was cultivated on a seawater medium enriched with 5 mM KNO_3, 0.2 mM KH_2PO_4, 1.5 μM $FeCl_3$, 30 μM EDTA, 5 mM $NaHCO_3$, 20 mM Hepes buffer (pH 7.5), trace metal mix (16), and NaCl at the indicated concentration.

Algae were grown in a constant temperature growth room under illumination with cool-white fluorescent lamps (light intensity, about 4 w m^{-2} at 23 C).

Assay of Glycerol. Three ml of *A. gracilis* suspension in the culture medium were transferred to a test tube and placed in a water bath maintained at about 70 C for 10 min (18). The samples were centrifuged at room temperature for 5 min at 2000*g* and the glycerol content of the supernatant was determined chemically as previously described (4). Enzymic determination of glycerol in the same supernatant (2) verified the results of this assay.

Cell Counting, Chl Determination, Specific Growth-rate Determination and Oxygen Evolution. Cells were counted in a Coulter counter, model ZB, with a 100-μm orifice tube. Chl was extracted from an algal pellet with 80% acetone and was assayed after Arnon (1). The specific growth rate (μ day^{-1}) was calculated from the logarithmic phase of the growth curve. A specific growth of 2 represents doubling time of the algae every 24 h. O_2 evolution and uptake were measured with a constant temperature Rank Brothers O_2 electrode connected to an Omniscribe recorder. The sample was illuminated by white light with an intensity of 54 w m^{-2} for O_2 evolution measurements.

RESULTS

Absorption Spectrum of *A. gracilis*. The absorption spectrum of 80% acetone extract of *A. gracilis* is similar to that of other green algae (Fig. 1), with Chl *a*, Chl *b*, and carotenoids prominent. The lack of a cell wall facilitates the direct extraction of pigments from the algae with no need for pretreatment.

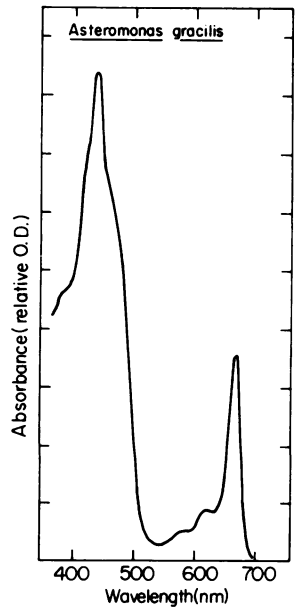

FIG. 1. Absorption spectrum of an 80% acetone extract of *A. gracilis*. Concentrated algae were extracted with 80% (v/v) acetone and measured in a Cary 16 spectrophotometer.

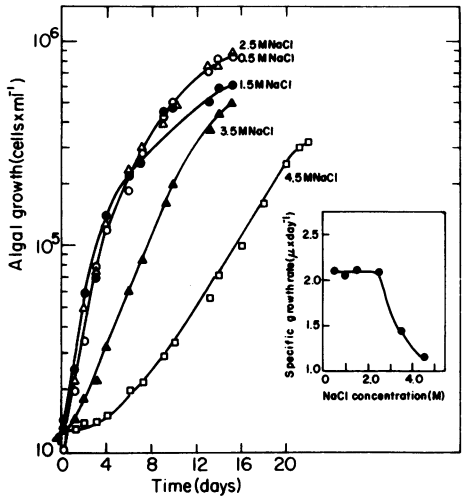

FIG. 2. The effect of the NaCl concentration of the medium on the growth and the specific growth rate of *A. gracilis*. A specific growth rate of 2 represents doubling time of the alga every 24 h.

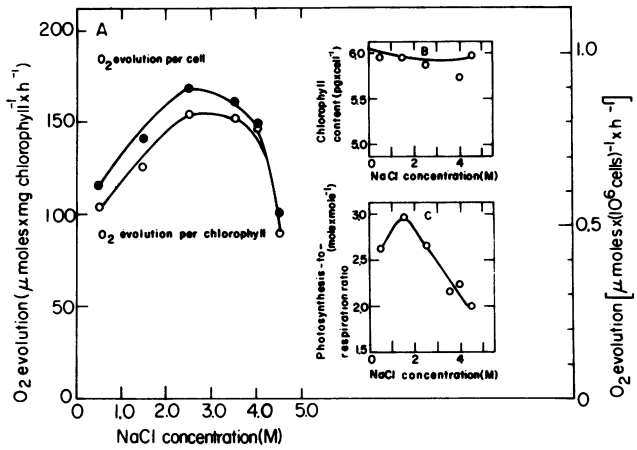

FIG. 3. The effect of the NaCl concentration of the medium on photosynthesis (A), Chl content (B), and photosynthesis to respiration ratio (C). Algae were grown in a medium containing the indicated salt concentration. At the logarithmic phase, the algae were concentrated and assayed for O_2 evolution and uptake at a final concentration of about 50 μg Chl/3 ml. C was plotted from the ratio of light O_2 evolution plus dark O_2 uptake/dark O_2 uptake.

Growth Characteristics. Cells that were originally obtained from the culture collection in seawater medium containing about 0.5 M NaCl were transferred gradually to various concentrations of NaCl. After adaptation and growth of several weeks in different concentrations of NaCl, the cells were assayed for growth by cell counting. Figure 2 illustrates that a remarkable adaptation to salt occurs in *A. gracilis*. Algae grown on salt concentrations between 0.5 to 2.5 M multiply every 24 h. Above 2.5 M, the growth rate gradually decreases to about 1.2 at 4.5 M NaCl. *Asteromonas* does not require extreme concentrations of salt for growth but is able to withstand saturated salt solutions. This indicates that it is halotolerant, rather than halophilic, as was previously shown for halophilic bacteria (12).

Effect of Salt on Photosynthesis and Respiration. Figure 3 shows that algae grown at various salt concentrations and assayed for photosynthetic O_2 evolution had a broad optimum at about 150 μmol O_2 mg^{-1} Chl h^{-1} around 2.5 M NaCl. This corresponds to about 0.75×10^{-6} μmol O_2 $cell^{-1}$ h^{-1}. At lower and higher NaCl concentrations, the decrease in O_2 evolution was relatively small, with lowest activity of about 90 μmol O_2 mg^{-1} Chl h^{-1} at 4.5 M NaCl. The slight differences between photosynthetic activity measured per cell and per Chl are due to the slight effect of salt on the Chl to cell ratio, which is approximately 6.0 pg/cell in the illustrated experiments (Fig. 3B). The photosynthesis to respiration ratio was a maximum of about 3 at 1.5 M NaCl (Fig. 3C). At salt concentrations above 1.5 M, the ratio of photosynthesis to respiration decreased gradually to about 2.0 at 4.5 M NaCl.

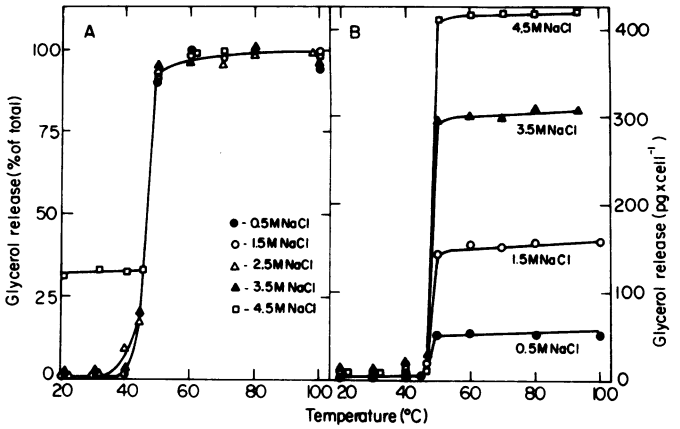

FIG. 4. Temperature dependence of glycerol release from *A. gracilis*. *A. gracilis* (3 ml) from cultures in logarithmic phase containing about 5 × 10^5 cells/ml were placed in a water bath maintained at the indicated temperature for 10 min. A, per cent of total extracellular and intracellular glycerol; B, intracellular glycerol content after deduction of the free glycerol present in the growth medium.

Glycerol Concentration in *A. gracilis*. Algae incubated for 10 min in growth media at different temperatures (18) released increasing amounts of glycerol to the external medium (Fig. 4A). At 47 C, 50% of intracellular glycerol leaked out of the alga. Above 60 C, all the glycerol was released from the cells. The amount released is proportional to the salt concentration in which the algae were grown (Fig. 4B). The temperature-release pattern and the temperature of 50% glycerol release were not affected by the salt concentration. Essentially, no release of glycerol occurred below 40 C; however, a small fraction of glycerol leaked during growth to the medium at salt concentration of 4.5 M NaCl (Fig. 4A).

For calculating the actual glycerol content in cells grown on 4.5 M NaCl, the extracellular glycerol was subtracted from total glycerol (Fig. 4B). A linear relation between intracellular glycerol and extracellular salt is observed over a range of salt concentrations from 0.5 to 4.5 M (Fig. 5A). The slight differences between the glycerol content per Chl and the glycerol content per cell are attributed to the variations in Chl content in algae grown at different salinities (Fig. 5B). In many experiments, like the one summarized in Figure 5B, the amount of Chl per cell in *A. gracilis* was around 5 pg/cell with an occasional tendency to increase with the salt concentration to around 6 pg/cell. The slight increase in Chl to cell ratio, and the tendency of glycerol per cell to depart from the linear relation with regard to salt concentration, were usually accompanied by a cell volume increase, as observed microscopically.

Kinetics of Glycerol Changes. When algae grown in 1.5 M NaCl were transferred to fresh media containing 2.5 and 3.5 M NaCl in the light at 23 C, about 5 h were required for the glycerol to reach a new equilibrium (Fig. 6A). When the reverse was done, glycerol content decreased and about 3 h were required to re-establish the equilibrium between the intracellular and extracellular conditions (Fig. 6B).

Both photosynthesis and respiration were inhibited temporarily when *A. gracilis* was transferred from 1.5 to 3.5 M NaCl (Fig. 7A). The kinetics of the resumption of photosynthesis and respiration

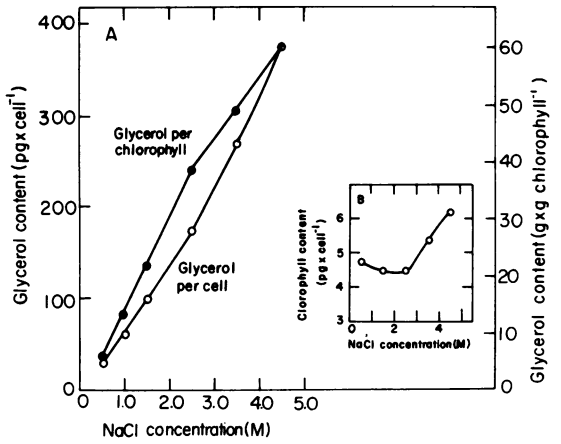

FIG. 5. The effect of extracellular NaCl concentration on the intracellular glycerol content in *A. gracilis*. Cells from the logarithmic phase were assayed for glycerol content. Fig. 5B illustrates the Chl content in cells assayed in this specific experiment.

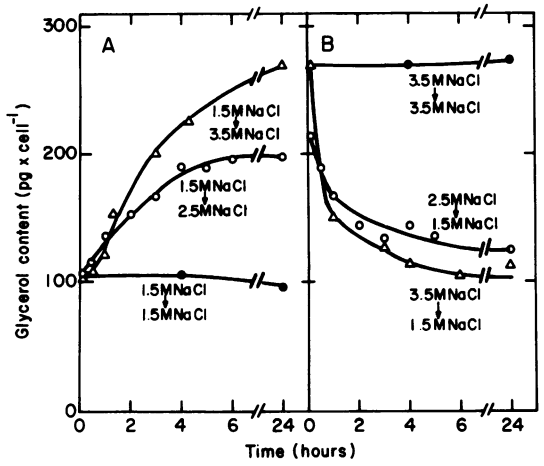

FIG. 6. Kinetics of increase and decrease of glycerol in *A. gracilis* in response to changes in the salt concentration of the medium. Algae from the logarithmic phase (containing about 5×10^5 cell/ml) from 1.5 M (A) or 3.5 M (B) NaCl were diluted or resuspended to obtain the indicated salt concentration and then were maintained under light at 23 C.

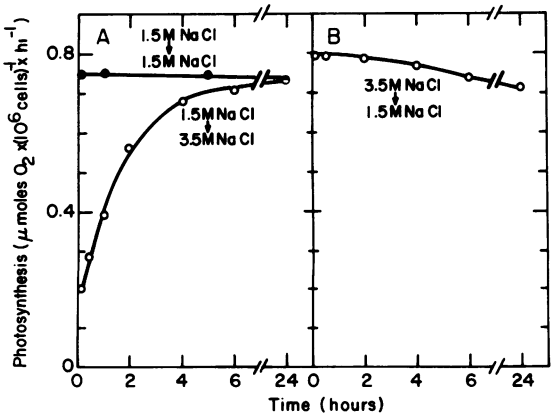

FIG. 7. The effect of hypertonic and hypotonic treatments on photosynthesis in *A. gracilis*. Experimental conditions were as described in Figure 6.

in *A. gracilis* preceded the kinetics of glycerol biosynthesis and were completed in about 3 h. The photosynthesis to respiration ratio changed accordingly from about 3 to 2.2. When *A. gracilis* grown in 3.5 M NaCl was transferred from 3.5 to 1.5 M NaCl, neither photosynthesis nor respiration were impaired (Fig. 7B).

DISCUSSION

The broad optimum in salt concentration for growth in *A. gracilis* is similar to that of the halotolerant alga *Dunaliella* (4, 6, 7) but quite different from that of halophilic bacteria (12). Both *Dunaliella* and *Asteromonas* grow over a full spectrum of salinities, from those of seawater to saturated solutions. There is no apparent requirement for high salt concentrations for growth but, rather, halotolerance.

Further evidence of halotolerance was observed in the values of photosynthesis and photosynthesis to respiration ratio which were only moderately affected by the salinity of the medium. Photosynthetic activities of *A. gracilis*, around 150 μmol mg^{-1} Chl h^{-1}, are similar to those of other nonhalotolerant unicellular algae (11). However, the photosynthesis to respiration ratio of about 2.5 found in the experiments presented here is lower than values around 10 found in *Dunaliella* and other marine algae (11). The reason for such a low value is not clear and warrants further experiments.

The photosynthetic activity and the specific growth rate of *A. gracilis* responded in a different manner to the salt concentration. Although the photosynthetic activity had a wide optimum, the specific growth rate dropped significantly at salt concentrations above 2.5 M NaCl. It was of interest to test whether the cells growing on increasing salt concentrations would show an increasing demand for photosynthetic energy for growth and maintenance. Figure 8 illustrates an increasing demand for photosynthetic activity to obtain a constant growth rate at elevated salinities. The results are interpreted as indicating the existence of an energetic competition between the demand of photosynthetic energy for growth and for production and accumulation of glycerol. A significant proportion of energy seems to be funnelled to glycerol synthesis in response to increasing salt augmentation. Above 4.0 M NaCl, the efficiency of the metabolic machinery of *Asteromonas* decreases, keeping the cells alive but at a very low growth rate.

The above data indicate that *A. gracilis* produces and accumulates large amounts of glycerol within the cell. The linear relation

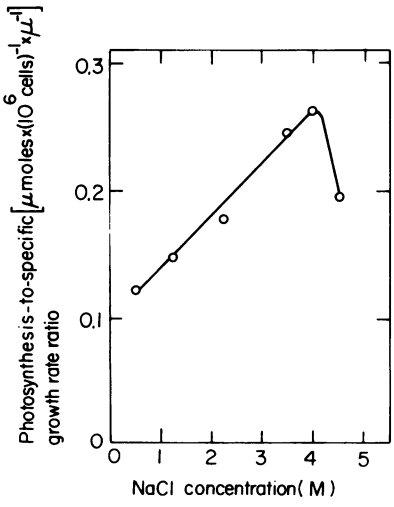

FIG. 8. The effect of NaCl concentration on the photosynthetic energy demand per unit of growth rate. The ratios of photosynthesis to specific growth rate were calculated from the photosynthetic values per cell in Figure 3 and from the specific growth rate (μ day^{-1}) in Figure 1.

of intracellular glycerol to the external salt concentration suggests that the major function of glycerol is to maintain the osmotic balance within the cell. The amount of glycerol per cell in *A. gracilis*, as in various species of *Dunaliella*, is related to the cell volume (5). Irrespective of cell volume, species of *Dunaliella* and *A. gracilis* of different volumes reach similar values of glycerol to Chl ratios, which are linearly related to the external salt concentration. The response of the glycerol to Chl ratio to salinity in *A. gracilis* confirms previous conclusions that internal glycerol serves as the major solute which osmotically balances the external salt concentration (2, 4, 5, 7).

Microscopic observations showed that *Asteromonas* cells behave like osmometers and rapidly shrink or swell under hypertonic or hypotonic conditions, respectively. Thus, *Asteromonas* cells lacking a rigid cell wall are able to adjust their osmotic pressure to the environment by rapid water movement through the cytoplasmic membrane. Thereafter, the cells return slowly to their original fluted pear-like volume through a unique metabolic adjustment. Under hypertonic conditions, the algae produce and accumulate intracellular glycerol until the new equilibrium is achieved. Under hypotonic conditions, glycerol decreases to the lower requirement of internal glycerol. Either way, water flux through the cytoplasmic membrane follows the new level of intracellular glycerol so that, at steady state, the original cell volume is regained.

As a consequence of the salt stress, both photosynthesis and respiration diminished significantly and resumed their normal level in a manner similar to, but preceding, that of the kinetics of the glycerol biosynthesis. The fate of the metabolic conversions of glycerol in *A. gracilis* is not clear. However, it is reasonable to suggest a metabolic conversion of starch to glycerol in the absence of photosynthesis and respiration. Preliminary observations (I. Sussman, unpublished) showed the presence of dihydroxyacetone reductase and dihydroxyacetone kinase in extracts of *A. gracilis*. Both enzymes are known to participate in the metabolic production and degradation of glycerol in *Dunaliella* (3, 13, 14). The presence of these unique enzymes in *A. gracilis*, and the kinetics of glycerol synthesis and photosynthesis resumption upon transition from low to high salt concentration, indicate that glycerol synthesis must in the first instance depend on polysaccharides such as starch. Thus, the immediate osmoregulatory response to an increase in salt concentration in the medium should involve the conversion of storage polysaccharides to glycerol. Such conversion requires a supply of ATP and NADPH, neither of which are supplied by photosynthesis or respiration due to the inhibition of both on hypertonic shock. Assuming an alternative source of energy and reducing power from glycolysis, then the hypothetical scheme of the osmoregulatory metabolism in *A. gracilis* may resemble that of *Dunaliella* (4, 5). In that scheme, the bioconversion of starch to glycerol proceeds through the production of triose-P, followed by reduction to glycerol phosphate and finally dephosphorylation. Conversion of glycerol to polysaccharides may proceed via oxidation of glycerol to dihydroxyacetone and phosphorylation to dihydroxyacetone phosphate.

The following points are made to summarize common features of *Asteromonas* and *Dunaliella*, two algae which are structurally and morphologically different. (*a*) Both algae lack a cell wall; the cells are enclosed by a thin elastic cell envelope. This permits rapid changes in cell shape and makes the cell highly responsive to osmotic changes. (*b*) Natural habitats of the halotolerant algae include salt pans, brine lakes, small pools, and salt water ditches near the sea in media containing salt in concentrations ranging from dilute to saturated. (*c*) Both algae have the unique property of maintaining an extreme concentration gradient of glycerol across the cell with little or no leakage into the medium. Glycerol concentration in the cells is proportional to the salt concentration in the growth medium. (*d*) Both algae release intracellular glycerol above a critical temperature of 47 C, suggesting a temperature-dependent conformational transition of a component of the cytoplasmic membrane which is essential for glycerol impermeability. (*e*) Both flagellates contain the enzymes dihydroxyacetone reductase and dihydroxyacetone kinase, two unique enzymes which are involved in the osmoregulatory response and have not been described elsewhere. Clearly, these common characteristics of *Asteromonas* and *Dunaliella* extend the scope of osmoregulation among naked algal cells.

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